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Reference(s):

1. FOCUS on Applications, Opti-MEM® I Reduced Serum Medium: Application in Tra Bulletin.

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REVIEW

Extracellular Regulation of BMP Signaling
in Vertebrates: A Cocktail of ModulatorsWendy Baemans and Wim Van Hul¹Department of Medical Genetics, University of Antwerp and University Hospital,
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The transforming growth factor- β (TGF- β) superfamily contains a variety of growth factors which all share common sequence elements and structural motifs. These proteins are known to exert a wide spectrum of biological responses on a large variety of cell types in both vertebrates and invertebrates. Many of them have important functions during embryonic development in pattern formation and tissue specification, and in adult tissues, they are involved in processes such as wound healing, bone repair, and bone remodeling. The family is divided into two general branches: the BMP/GDF and the TGF- β /Activin/Nodal branches, whose members have diverse, often complementary effects. It is obvious that an orchestrated regulation of different actions of these proteins is necessary for proper functioning. The TGF- β family members act by binding extracellularly to a complex of serine/threonine kinase receptors, which consequently activate Smad molecules by phosphorylation. These Smads translocate to the nucleus, where they modulate transcription of specific genes. Three levels by which this signaling pathway is regulated could be distinguished. First, a control mechanism exists in the intracellular space, where inhibitory Smads and Smurfs prevent further signaling and activation of target genes. Second, at the membrane site, the pseudoreceptor BAMBI/Nma is able to inhibit further signaling within the cells. Finally, a range of extracellular mediators are identified which modulate the functioning of members of the TGF- β superfamily. Here, we review the insights in the extracellular regulation of members of the BMP subfamily of secreted growth factors with a major emphasis on vertebrate BMP modulation. © 2002 Elsevier Science (USA)

Key Words: BMP; signaling; BMP antagonism; extracellular modulation.

INTRODUCTION

Bone morphogenetic proteins (BMPs) are secreted growth factors, which, based on amino acid homology of a highly conserved seven-cysteine domain in the carboxy-terminal region of the proteins, form a subgroup of the transforming growth factor- β (TGF- β) superfamily (Kingsley, 1994). They are dimeric proteins with a single interchain disulfide bond, and this dimeric conformation is an absolute requirement for the biological action of BMPs (Eimon and Harland, 1999). BMPs were originally isolated by their ability to induce ectopic bone and cartilage formation *in vivo* (Urist, 1965), but it became rapidly evident that BMPs also act as multifunctional regulators in morphogenesis during development in vertebrates and invertebrates (reviewed in Hogan, 1996a,b; Graff, 1997; Ebendal *et al.*, 1998; Wozney,

1998). More than 30 BMP family members have already been described. They have been classified into several subgroups according to their structural similarities. Individually, the members of this TGF- β subfamily are termed BMPs, osteogenic proteins (OPs), cartilage-derived morphogenetic proteins (CDMPs), and growth and differentiation factors (GDFs) (Ducy and Karsenty, 2000), and also include BMP-like molecules from various species, including the fly *Drosophila melanogaster* [decapentaplegic (dpp); 60A-glass bottom boat (gbb); and Screw] and the frog *Xenopus laevis* (Vg1). In the nematode *Caenorhabditis elegans*, three BMP-like pathways have been characterized: the dauer; Sma/Mab; and unc-129 pathways (Ren *et al.*, 1996; Colavita *et al.*, 1998; Suzuki *et al.*, 1999).

BMP SIGNALING PATHWAY

BMPs are synthesized as large precursor proteins. Upon dimerization, the molecules are proteolytically cleaved

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TABLE 1

Type I and Type II Serine/Threonine Kinase Receptors Involved in the Signaling Pathway of TGF- β Superfamily Members and Their Ligands

Serine/threonine kinase receptor	Ligand
Type I receptors or activin receptor-like kinases (ALK)	
ALK-1	BMPs
ALK-2 or ActR-I	BMPs, Activin
ALK-3 or BMPR-IA	BMPs
ALK-4 or ActR-IB	TGF- β , Activin
ALK-5 or T β R-I	TGF- β , Activin
ALK-6 or BMPR-IB	BMPs, AMH
ALK-7	?
Type II receptors	
Activin type II (ActR-II)	Activin, BMPs
Activin type IIB (ActR-IIB)	Activin, BMPs
TGF- β type II receptor (T β R-II)	TGF- β
BMP type II receptor (BMPR-II)	BMPs
Anti-müllerian hormone type II receptor (AMHR-II)	AMH

within the cell to yield carboxy-terminal mature proteins. Once secreted, the BMP dimers initiate signaling by binding cooperatively to both type I and type II serine/threonine kinase receptors (Ducy and Karsenty, 2000; Miyazono *et al.*, 2000). The type II receptors are constitutively active kinases, which transphosphorylate type I receptors upon ligand binding. The type I receptors activate intracellular substrates by phosphorylation, and thus determine the specificity of intracellular signals (Fig. 1). In mammals, only seven type I receptors and five type II receptors have been identified thus far (reviewed in Kawabata *et al.*, 1998; Miyazono *et al.*, 2000) (Table 1). Signals from the serine/threonine kinase receptors may be transduced by various proteins. Genetic studies using *C. elegans* and *Drosophila* have resulted in the identification of these downstream signaling mediators of the receptors for BMP-like ligands in these organisms, which were called, respectively, *sma* (Savage *et al.*, 1996) and *Mothers against dpp* (*Mad*) (Sekelsky *et al.*, 1995). An increasing number of the vertebrate members belonging to this protein family have subsequently been identified and are denoted Smad, a fusion of *sma* and *Mad* (reviewed in Derynck *et al.*, 1998; Raftery and Sutherland, 1999; Kawabata *et al.*, 1999). Eight Smads are known in mammals and are classified into three groups based on their structures and functions. Receptor-regulated Smads (R-Smads) transiently associate with type I serine/threonine kinase receptors and undergo direct phosphorylation. Smad1, -5, and -8 are activated by BMPR-IA or BMPR-IB (Hoodless *et al.*, 1996; Nishimura *et al.*, 1998; Kawai *et al.*, 2000), whereas Smad2 and -3 mediate TGF- β signaling (Macías-Silva *et al.*, 1996; Zhang *et al.*, 1996a). These R-Smads associate with the common partner Smad (Co-Smad or Smad4), and these heteromeric complexes translo-

cate to the nucleus, where they interact with other transcription factors to regulate the transcription of target genes (Fig. 1). Finally, Smad6 and Smad7 interfere with R-Smads and Co-Smads and are called inhibitory Smads (I-Smads) (Imamura *et al.*, 1997; Casellas and Brivanlou, 1998). Besides signal transduction through Smad molecules, other pathways may also be involved in the generation of signals by members of the TGF- β superfamily, like MAP kinases, which are activated by BMPs and TGF- β s in certain cell types, as reviewed in Mulder (2000).

FUNCTIONS OF BMPs

As previously mentioned, the first BMPs were originally isolated by their ability to induce ectopic bone and cartilage formation *in vivo* in muscle tissue or subcutaneous sites of rodents (Urist, 1965). This unique bone-inductive activity indicates that BMPs provide the primordial signals for osteodifferentiation. It is known that BMP proteins initiate the cascade of endochondral bone formation, where mesenchymal stem cells differentiate into chondrocytes which lay down a cartilage structure that is reabsorbed and replaced by (reviewed in Reddi, 1994) bone tissue. BMPs also act as local factors in the regulation of osteoblast differentiation as demonstrated in previous experiments (Katagiri *et al.*, 1990, 1994; Yamaguchi *et al.*, 1991; Sampath *et al.*, 1992; Gitelman *et al.*, 1995; and others). However, BMP expression studies, as well as the analysis of BMP mouse models, have demonstrated a much broader range of biological activities of the different BMP family members on various cell types, including monocytes, epithelial cells, mesenchymal cells, and neuronal cells. BMPs regulate cell proliferation and differentiation, chemotaxis and apoptosis, and control fundamental roles such as left-right asymmetry, neurogenesis, mesoderm patterning, and development of a number of organs, such as kidney, gut, lung, teeth, limb, amnion, and testis (reviewed in Hogan 1996a,b; Graff, 1997; Ebendal *et al.*, 1998; Wozney, 1998). Several BMP knockout experiments in mice have contributed to elucidate the role of BMPs at different stages of development. *BMP-2* deficient mice had amnion/chorion malformation and defects in cardiac development, and died during embryonic development (Zhang *et al.*, 1996b). A skeletal phenotype was seen in adult *BMP-3*^{-/-} mice. These mice demonstrated an increased trabecular bone density of the metaphysis of the femur, indicating that BMP-3, in contrast to the other BMP family members, is a negative determinant of bone density (Daluiski *et al.*, 2001). A null mutation of the *BMP-4* gene in mice resulted in defects in the extraembryonic and posterior/ventral mesoderm formation and are also embryonic lethal (Winnier *et al.*, 1995). The recessive *short ear* (*se/se*) mice have mutations in the *BMP-5* gene and demonstrated abnormalities in the skull and axial parts of the skeleton, and showed numerous tissue abnormalities, affecting lung, liver, uterus, bladder, and intestine. These *BMP-5* null mice were viable and fertile

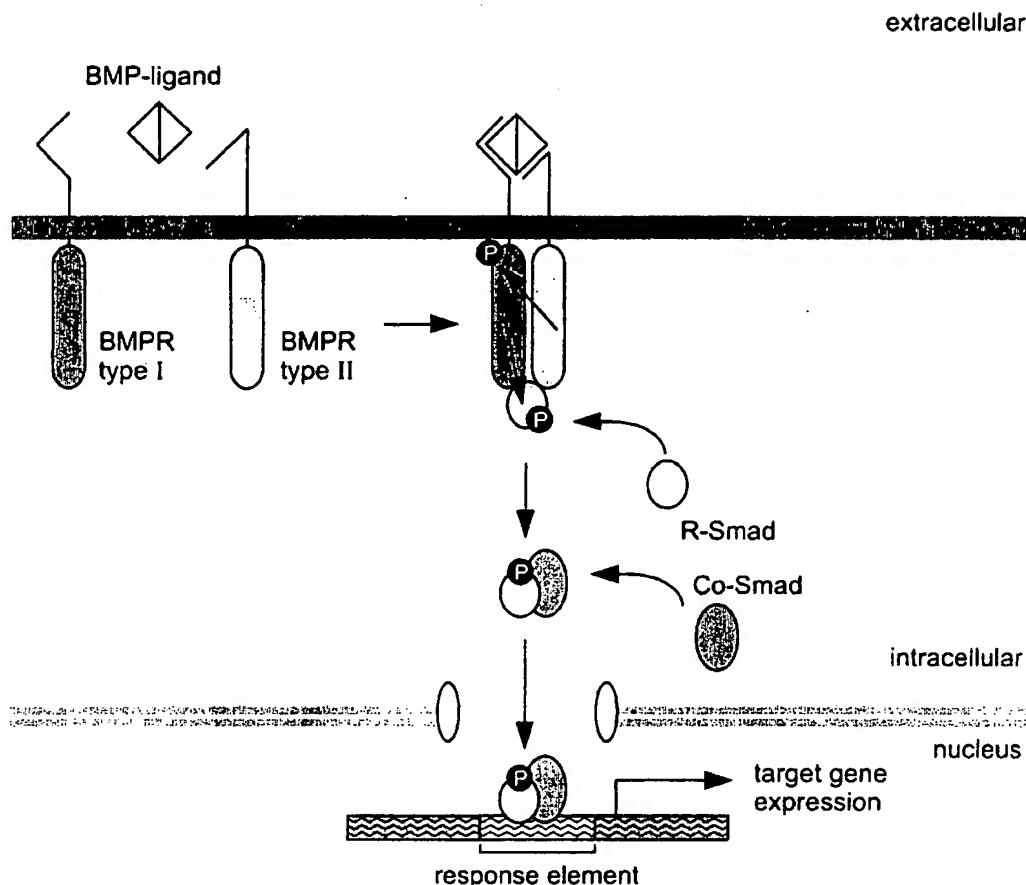


FIG. 1. Cascade of BMP signaling. BMP dimers bind to serine/threonine kinase receptors type I and II. Upon ligand binding, type II receptors transphosphorylate type I receptors. The latter phosphorylate members of the Smad family of transcription factors. These Smads are subsequently translocated to the nucleus, where they activate transcription of target genes.

(Green, 1958; Kingsley *et al.*, 1992; King *et al.*, 1994). Targeted inactivation of *BMP-6* in mice resulted in viable and fertile mice, with no overt defects in tissues known to express *BMP-6*; however, careful examination of skeletogenesis in late gestation embryos revealed a mild delay of sternum ossification (Solloway *et al.*, 1998). *BMP-7* knockout mice were born alive with polydactyly of the inner digit of the hindlimbs, and most of them died within 24 h after birth. The mice showed skeletal defects, with a smaller overall size compared with their normal littermates, and suffered from defects in eye development. The kidney was small and showed poor nephron development (Jena *et al.*, 1997). Mice with mutations in the *GDF-5* gene, also known as *brachypodism* (*bp*) mice, have short limbs, altered formation of bones and joints in the sternum, and reduced numbers of bones in the digits (Storm *et al.*, 1994). Targeted inactivation of *BMP-8B*, a gene present in mice but absent in humans, resulted in abnormalities in spermatogenesis in early puberty and in adulthood (Zhao *et al.*, 1996).

MODULATION OF BMP SIGNALING

Finely tuned signals between cells are necessary to coordinate all aspects of development, from patterning of the embryonic body axis to homeostasis of adult tissues. For this reason, BMP signaling is subjected to delicate regulation at multiple levels: intracellularly, at the membrane site, and extracellularly (Fig. 2). The first level of regulation resides within the cell cytoplasm, where inhibitory Smads and Smurfs act as modulators. Von Bubnoff and Cho (2001) wrote a comprehensive review on this intracellular regulation of BMP signaling in vertebrates. Recently, the transmembrane protein BAMBI (BMP and Activin membrane-bound inhibitor), playing a role in attenuating BMP signaling, was identified in *Xenopus*. BAMBI encodes a TGF- β pseudoreceptor, which stably associates with TGF- β -family receptors and interferes with BMP, Activin, and TGF- β signaling (Onichtchouck *et al.*, 1999; Grotewold *et al.*, 2001). Finally, in the extracellular space, a control

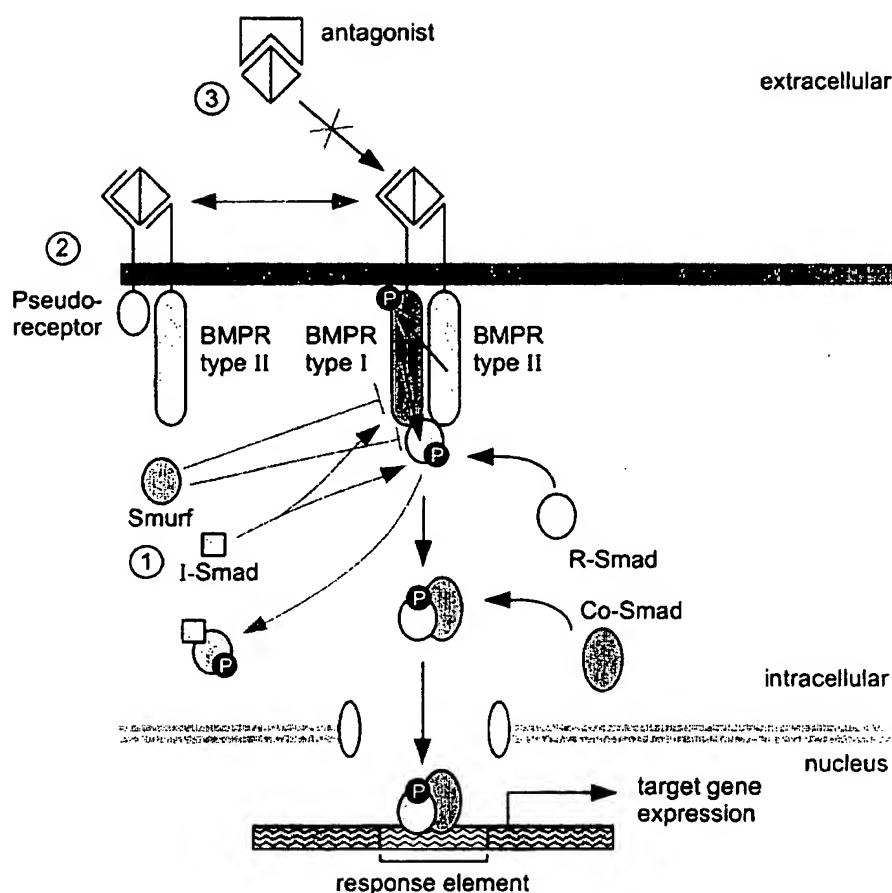


FIG. 2. Three levels of modulation of BMP signaling. (1) I-Smads and Smurfs regulate intracellular signaling by preventing further Smad signaling and consequent activation of gene transcription, (2) Pseudoreceptor BAMBI/Nma modulates BMP signaling at the membrane site by binding to BMP type II receptors, and (3) Extracellular antagonists modulate binding of BMP dimers to the BMP type I and type II receptors.

system exists, whereby high affinity antagonists complex selectively with BMPs to inhibit their biological activities.

Intracellular Modulation

Inhibitory Smads (I-Smads), i.e., Smad6 and Smad7, antagonize the TGF- β signaling pathway either by interacting with phosphorylated type I receptors and thereby preventing the activation of R-Smads, or through competition with Co-Smads for the formation of the R-Smad/Co-Smad complexes (Itoh *et al.*, 2001) (Fig. 2). The expression of *Smad6* and *Smad7* is induced by multiple stimuli, including epidermal growth factor (EGF) and various TGF- β family members, such as TGF- β 1, Activin, and BMP-7, suggesting the existence of a negative feedback signal (Nakao *et al.*, 1997). It has also been demonstrated that *Smad6* and *Smad7* expression is regulated by pathological conditions, such as laminar shear stress in vascular endothelial cells (Topper *et al.*, 1997).

Two closely related Smurfs (Smad ubiquitination regulatory factors), Smurf1 and Smurf2, have been identified in vertebrates and are mediators of the final step in ubiquitination of target proteins (Zhu *et al.*, 1999a, 2001). This family of enzymes, which contains HECT catalytic domains characteristic of E3-ubiquitin ligases, modulate TGF- β signaling by selectively targeting the activated type I receptors (Kavsak *et al.*, 2000; Ebisawa *et al.*, 2001) and Smad proteins (Lo and Massagué 1999; Lin *et al.*, 2000; Zhang *et al.*, 2001) for degradation (Fig. 2).

Membrane Receptor Modulation

Xenopus BAMBI is a pseudoreceptor for members of the TGF- β superfamily showing high sequence similarity to Nma, its mammalian homologue. BAMBI/Nma is structurally related to type I serine/threonine kinase receptors in the extracellular domain, but it lacks the intracellular serine/threonine kinase domain (Onichtchouck *et al.*, 1999;

Grotewold *et al.*, 2001). The pseudoreceptor antagonizes the effects of TGF- β , Activin, and BMPs by stably associating with serine/threonine kinase receptors, thus preventing the formation of active receptor complexes (Onichtchouk *et al.*, 1999) (Fig. 2).

Extracellular Modulation

The exact concentration of active TGF- β s seems to be important for rendering a particular biological effect. It was found in *Xenopus* and *Drosophila* that an activity gradient of the members of the BMP family of growth factors is formed during embryogenesis, leading to the generation of different cell types, depending on the location (Wharton *et al.*, 1993; Dale and Wardle, 1999; Dale and Jones, 1999; De Robertis *et al.*, 2000). The active local concentration and consequently the extent of action of these morphogens is controlled, in part, by the influence of extracellular modulators (Barth *et al.*, 1999; Liem *et al.*, 2000; Hama and Weinstein, 2001) (Fig. 2). An increasing number of these natural antagonists is being identified in vertebrates and invertebrates, and appears to have evolved independently due to lack of primary sequence conservation. In vertebrates, the list of BMP antagonists already comprises more than seven proteins, including noggin, chordin, chordin-like, follistatin, FSRP, the DAN/Cerberus protein family, and sclerostin. Studies in *Drosophila* demonstrated the presence of similar antagonists in invertebrates. The *Drosophila* short gastrulation (*sog*) is the homologue of chordin, and affects dorsal-ventral patterning by antagonizing *dpp* and *screw*, the homologues of, respectively, vertebrate BMP-2/-4 and BMP-5/-6/-7/-8 (Holley *et al.*, 1995; Neul and Ferguson, 1998). In both vertebrates and invertebrates, other regulating factors are involved in the BMP/*dpp*-*screw* pathway. Secreted zinc metalloproteinases antagonize the activity of chordin/*sog*, including *Drosophila* Tolloid (Tld), *Xenopus* Xolloid (Xol), and human BMP-1 (Marques *et al.*, 1997). Another extracellular factor, twisted gastrulation (*tsg*), which is conserved among vertebrate and invertebrate species, influences this proteinase cleavage and modifies the interactions between chordin/*sog* and BMP/*dpp*-*screw* by binding to the complex. The formation of this ternary complex leads to a more efficient inhibition of BMP/*dpp* activity (Oelgeschläger *et al.*, 2000). The goal of this review is to focus on the group of the vertebrate extracellular BMP antagonists.

REGULATION OF BMP SIGNALING IN VERTEBRATES THROUGH EXTRACELLULAR MODULATORS

Noggin

Noggin was first isolated from *X. laevis* based upon its ability to rescue dorsal development in embryos ventralized by UV treatment (Smith and Harland, 1992) and is expressed during early stages of gastrulation in the Spemann

organizer, a tissue which plays a central role in specifying dorsal cell fates in both ectoderm (neural plate) and mesoderm (somite and notochord), where it promotes the development of dorsal tissues, such as muscle and nervous tissue (McMahon *et al.*, 1998).

In the zebrafish embryo, three noggin orthologues have been isolated, *noggin1*, *noggin2*, and *noggin3*, with *noggin1* and *noggin3* being highly related to the noggins of higher vertebrates and *noggin2* being somewhat more divergent. *Noggin1* is expressed in the organizing center of the zebrafish, while *noggin2* transcripts first appear at the end of gastrulation. Both genes are expressed during somitogenesis and during formation of the nervous system. *Noggin1* and *noggin2* overexpression strongly dorsalizes the embryo, suggesting a role in dorsoventral patterning of the zebrafish (Bauer *et al.*, 1998). *Noggin* expression is limited to late stages of embryonic development and is restricted to regions of ongoing chondrogenesis (Fürthauer *et al.*, 1999).

During the early stages of mouse embryonic development, *noggin* is expressed in the node, a structure with similar properties to the *Xenopus* organizer. However, knockout experiments in mice failed to show the requirement of *noggin* in the formation of neural tissue, notochord, or somites during the early gastrulation stages (McMahon *et al.*, 1998). Bachiller *et al.* (2000) were able to demonstrate in *noggin/chordin* double knockout mice, that, in these early stages of development, the loss of *noggin* function could be rescued by chordin, another BMP antagonist. They showed an overlap in expression of both genes at the midgastrula stage. Absence of both *noggin* and *chordin* expression led to inappropriate anteroposterior, dorsoventral, and left-right patterning (Table 2). Later on in mouse embryonic development, *noggin* expression was seen in the notochord, the roof of the neural tube, the dorsal aspect of the somites, and the limbs. Loss of *noggin* expression in these later sites has profound effects on development and results in a recessive lethal phenotype at birth. *Noggin* knockout mice are characterized by numerous defects: a shortened body axis caudal to the forelimbs with reduced size of the somites and neural tube, an open neural tube of varying severity, loss of caudal vertebra, and malformed limbs (McMahon *et al.*, 1998) (Table 2). Brunet *et al.* (1998) studied the same *noggin* knockout mouse model and showed that expression of the mouse *noggin* gene is essential for proper skeletal development. In these homozygous mutant mice, vertebra, ribs, and limbs were all affected. Examination revealed excess bone and cartilage and failure to initiate joint formation due to excess of BMP activity (Table 2). Absence of *noggin* in these mutant mice and the consequent absence of regulating BMP activity probably lead to failure of activating the *GDF-5* gene, necessary to initiate joint development. *Noggin* has been proposed as an upstream modulator of GDF-5 signaling, which is consistent with the similar pattern of joints affected in *noggin* and *GDF-5* (*brachypodism*, *bp*) null mice (Storm *et al.*, 1994; Storm and Kingsley, 1996; McMahon *et al.*, 1998; Brunet *et al.*, 1998). Kulesa *et al.* (2000) used transgenic mice over-

TABLE 2
Mouse Models Involving the Different BMP Antagonists

Model	Type	Phenotypical abnormalities	Reference
Noggin ^{-/-}	knockout	lethal phenotype shortened body axis reduced size of somites and neural tube loss of caudal vertebra malformed limbs excess bone and cartilage failure to initiate joint formation	McMahon <i>et al.</i> (1998); Brunet <i>et al.</i> (1998)
Noggin	Transgenic (Msx2 promoter)	defects in postnatal hair development	Kulesa <i>et al.</i> (2000)
Chordin ^{-/-}	knockout	limb abnormalities stillborn animals defects in inner and outer ear development abnormalities in pharyngeal and cardiovascular organization	Bachiller <i>et al.</i> (2000)
Chordin-like Noggin ^{-/-} /Chordin ^{-/-}	—	—	—
Follistatin ^{-/-}	knockout	defects in antero-posterior, dorso-ventral and left-right patterning lethal phenotype reduced size skeletal abnormalities defects in whisker and tooth development shiny, taut skin	Bachiller <i>et al.</i> (2000) Matzuk <i>et al.</i> (1995)
Follistatin	Transgenic (MT-I promoter)	reduced fertility smaller testis in males smaller ovaries and thinner uteri in females minor defects in hair formation	Guo <i>et al.</i> (1997)
FSRP	Transgenic (MT-I promoter)	Severely impaired fertility	Schneyer <i>et al.</i> (2001)
Dan ^{-/-}	knockout	No obvious phenotypical abnormalities	Dionne <i>et al.</i> (2001)
Dan ^{-/-} /Noggin ^{-/-}	knockout	transformation of last lumbar vertebrae to sacral fate	Dionne <i>et al.</i> (2001)
Cerberus ^{-/-}	knockout	no obvious phenotypical abnormalities	Simpson <i>et al.</i> (1999)
Cerberus ^{-/-}	knockout	no obvious phenotypical abnormalities	Beio <i>et al.</i> (2000)
Cerberus ^{-/-}	knockout	no obvious phenotypical abnormalities	Shawlot <i>et al.</i> (2000)
Cerberus ^{lacZ/lacZ}	knockout	no obvious phenotypical abnormalities	Stanley <i>et al.</i> (2000)
Cerberus ^{-/-} /Noggin ^{-/-}	knockout	only defects due to lack of noggin	Borges <i>et al.</i> (2001)
Grem1	—	—	—
Dante	—	—	—
PRDC	—	—	—
Sclerostin	—	—	—
Sclerostin-like	—	—	—

expressing *noggin* under control of the mouse *Msx2* promoter to demonstrate the involvement of *noggin* in postnatal hair development. These mice form hair follicles but lack external hairs, due to impaired differentiation of shaft hair cells. Additionally, these mice display mild limb abnormalities (Table 2).

Noggin is known to bind and antagonize BMP-2, -4, and -7, with a higher affinity for BMP-2 and -4 (Zimmerman *et al.*, 1996). Studies demonstrated that *noggin* inhibits BMP-4 activity in a competitive manner by binding to BMP-4 and consequently interfering with the ability of BMP-4 to bind to cognate cell-surface receptors (Zimmerman *et al.*, 1996). Additionally, *noggin* has been shown to interact directly

with GDF-5 *in vitro* and influences its effect *in vivo*. Merino *et al.* (1999a) suggested a possible antagonistic role for *noggin* in regulating the signaling of GDF-5 during digit skeletogenesis in the embryonic chick bud (Table 3).

The *noggin* gene, encoding a secreted protein of 232 amino acids, was mapped to human chromosome 17q22 (Valenzuela *et al.*, 1995), in the region where both proximal symphalangism (SYM1; MIM185800) (Polymeropoulos *et al.*, 1995) and multiple synostoses syndrome (SYNS; MIM186500) (Krakow *et al.*, 1998) are located. Both conditions are characterized by multiple joint fusions. Evidence was provided that *noggin* is associated with joint and vertebra formation in humans by the identification of

TABLE 3

Ligands for the Different BMP Antagonists

	Comments	BMP-2	BMP-4	BMP-5	BMP-6	BMP-7	GDF-5	GDF-11	Activin	Nodal	Wnt
Noggin		++	++			+	+				
Chordin		++	++			+					
Chordin-like			+	+	+						
Follistatin		+	+			++		+	+++		
FSRP		+			+	++			+++		
DAN		+	+			+	++				
Cerberus		+	+			+			+	+	+
Gremlin		+	+			+					
Dante	Unknown										
PRDC	Unknown										
Sclerostin				+	+						
Sclerostin-like	Unknown										

disease-causing mutations in five unrelated families with SYM1, a mutation in an isolated patient with SYM1 and one mutation was detected in a family with SYNS (Gong *et al.*, 1999). *Noggin* is also involved in Fyrodysplasia Ossificans Progressiva (FOP; MIM135100), a rare genetic disorder characterized by congenital malformation of the great toes and progressive heterotrophic endochondral ossification of the muscles (Connor and Evans, 1982). A genome-wide linkage search localized the *FOP* gene to the human chromosome 17q21-q22 region, close to *noggin* (Lucotte *et al.*, 2000). Initially, a 42-bp deletion was found in one FOP patient (Lucotte *et al.*, 1999), and recently, three different missense mutations in *noggin* were detected in three Spanish families with FOP (Sermonin *et al.*, 2001). It has also been demonstrated that missense mutations in *noggin* cause tarsal/carpal coalition syndrome (TCC; MIM186400), a condition characterized by multiple carpal and tarsal synostosis, as well as radial-head distortion, aplasia, or hypoplasia of the middle phalanges, and metacarpophalangeal synostosis (Dixon *et al.*, 2001) (Table 4).

Chordin and Chordin-Like

Like *noggin*, *chordin* plays major roles in dorsoventral axis formation and in the induction, maintenance, and/or differentiation of neural tissues during gastrulation, and is secreted by the Spemann organizer of *Xenopus* and zebrafish, and by the node of chick and mouse embryos (Streit *et al.*, 1998; Sasai *et al.*, 1994; Miller-Bertoglio *et al.*, 1997). When *chordin* is secreted from the organizer, it acts by interfering in BMP signaling, allowing dorsally derived tissues, such as neurectoderm and somitic muscle, to develop (Miller-Bertoglio *et al.*, 1997). Genetic data, which support and extend the findings in *Xenopus*, have been obtained in zebrafish, where as a null mutation in the zebrafish *chordin*, designated *chordino*, showed a ventralized phenotype (Schulte-Merker *et al.*, 1997).

Chordin expression in the mouse is seen in most major

organs, like brain, lung, liver, and kidney, suggesting multiple functions in organogenesis. *Chordin* expression is relatively high in condensing and differentiating cartilage elements, where it is coexpressed with BMP-2, -4, and -7, indicating an important role in embryonic skeletogenesis (Scott *et al.*, 2000). Fisher and Halpern (1999) already provided evidence that *chordin* plays an important role in patterning of the axial skeleton in zebrafish. Targeted inactivation of *chordin* in mice resulted in stillborn animals, which have normal early development and neural induction, but at later stages of embryogenesis, they showed defects in inner and outer ear development and abnormalities in pharyngeal and cardiovascular organization (Bachiller *et al.*, 2000) (Table 2).

Chordin binds BMP-2, -4, and -7 in a way similar to *noggin*, with a higher affinity for BMP-2 and -4, and antagonizes BMP signaling by blocking the binding to the BMP receptors (Piccolo *et al.*, 1996) (Table 3).

Radiation hybrid mapping localized the human *chordin* gene to chromosome 3q27 (Pappano *et al.*, 1998). The gene encodes a protein of 955 amino acids, containing a signal peptide for secretion and four cysteine-rich (CR) domains. Recently, three alternatively spliced, human *chordin* variants have been described, carrying at least part of one of the four CR domains, and showing biological activities (Millet *et al.*, 2001) (Table 4). These results suggested a possible influence of these variants on BMP activity in different developmental situations. *Chordin* has been considered a candidate gene for Cornelia de Lange syndrome (CDLS; MIM122470), a condition characterized by typical facies in association with prenatal and postnatal growth retardation, mental retardation, and upper limb anomalies (de Lange, 1933). The *CDLS* gene was previously mapped to this chromosomal 3q27 region, but mutation analysis in patients with CDLS failed to reveal disease-causing mutations (Smith *et al.*, 1999).

Nakayama *et al.* (2001) isolated from mouse bone marrow stromal cells the *chordin-like* protein (CHL), which showed

TABLE 4

Number of Amino Acids, Chromosomal Localization, and Associated Disease, and Also Alternative Names for the Different Human BMP Antagonists

Protein	Alternative names	Amino acids	Chromosome	Associated disease
Noggin		232	17q22	Proximal symphalangism Multiple synostoses syndrome1 Fybrodysplasia ossificans progressiva Tarsal/carpal coalition syndrome
Chordin		955 86 (variant 1) 94 (variant 2) 350 (variant 3)	3q27	—
Chordin-like	Neuralin-1 (mouse) Ventroptin (chick)	CHL(11) 452 CHL(12) 451 CHL(13) 447 CHL(14) 446	Xq22.1-q23	—
Follistatin		344	5q11.2	—
FSRP		263	19p13	—
DAN		180	1p36.13-p36.11	—
Cerberus CER1	Caronte (chick)	267	9p22	—
Gremelin		184	15q13-q15	—
Dante		≥80	?	—
PRDC		168	1q42-q43	—
sclerostin		213	17q12-q21	Sclerosteosis van Buchem disease
Sclerostin-like		206	7p21	—

significant homology to chordin and interacts *in vitro* with BMP-4, -5, and -6. As a result of alternative splicing and alternative transcriptional termination, four forms of CHL exist in mouse and human, the short forms mCHL(s1) and mCHL(s2), and the long forms mCHL(11) and mCHL(12) in mice, and four long forms, CHL(11)–CHL(14), in humans. Coffinier *et al.* (2001) isolated a chordin-related molecule, called neuralin-1, which, based on amino acid sequence comparison, showed 100% homology with CHL, indicating that both groups isolated an identical protein. Additionally, another group isolated from chick retina a protein called ventroptin with a possible function in the determination of dorsal–ventral cell fates in the retina by antagonizing BMP-4 (Sakuta *et al.*, 2001). Amino acid sequence analysis provided evidence that this novel protein is identical with CHL. CHL (or *neuralin-1* or *ventroptin*) is expressed preferentially in mesenchymal cell lines, and the spatiotemporal expression in mice differs from that of *chordin*, suggesting that both proteins would have different and/or nonoverlapping biological roles. During mouse embryogenesis, CHL expression was first detected in the somites and neural plate, the dermatome, and limb bud mesenchymes. High expression levels were seen in the developing skeletal structures, including limb bones, clavicles, calvaria, vertebra, and ribs. In the adult mouse, CHL is expressed in brain, lung, kidney, and testis. Expression of CHL in nonskeletal mesenchymal cells was demonstrated in many adult connective tissue cell types (Nakayama *et al.*, 2001).

Follistatin and FSRP

Follistatin was originally isolated from ovarian fluid by virtue of its ability to suppress follicle-stimulating hormone (FSH) secretion from the pituitary. The follistatin protein has been isolated on the basis of its involvement during the reproductive cycle and is expressed in a wide range of embryonic and adult tissues, including brain, testis, ovary, bone marrow, placenta, and the anterior pituitary (reviewed in Patel, 1998).

Expression patterns of *follistatin* in the organizer of the blastopore in *Xenopus* embryos at the onset of gastrulation suggest that this gene might have a dorsal function in *Xenopus* embryos, in addition to its role as a neural inducer (Hemmati-Brivanlou *et al.*, 1994; Fainsod *et al.*, 1997).

Follistatin knockout mice display a number of defects. They are smaller than their heterozygous littermates and they present with less muscle. The mice fail to breathe and die soon after birth. They also display certain skeletal abnormalities as well as improper tooth and whisker development, and their skin is taut and shiny (Matzuk *et al.*, 1995). Transgenic mice overexpressing *follistatin*, under control of the mouse *metallothionein* (MT)-I promoter, were not lethal and were of normal size, but had shiny irregular fur. Fertility was reduced: males had smaller testis whereas females had smaller ovaries and thinner uteri, clearly indicating a key role for follistatin during sexual development (Guo *et al.*, 1998) (Table 2).

Follistatin was identified as an Activin-binding protein that prevents Activin from binding to its receptor (Nakamura *et al.*, 1990; Hemmati-Brivanlou *et al.*, 1994). It has also been shown that follistatin binds BMP-2, -4, and -7, with higher affinities for BMP-7 (Yamashita *et al.*, 1995; Iemura *et al.*, 1998), although the affinity of follistatin for BMPs is lower than that of follistatin for Activin (Nakamura *et al.*, 1990; Iemura *et al.*, 1998). Iemura *et al.* (1998) demonstrated that follistatin can inhibit the effect of BMP-2, -4, and -7 in a manner different from that of the other organizer factors, noggin and chordin, by binding to BMP receptors through BMPs, forming a trimeric complex. Recently, Camer *et al.* (2001) demonstrated antagonistic action of follistatin for GDF-11, a key regulator of patterning the axial skeleton in chick limb buds (Table 3).

Activin signaling has been implicated in early limb development (Stern *et al.*, 1995) and in skeletal muscle differentiation (Link and Nishi, 1997), and probably also in limb skeletogenesis. Merino *et al.* (1999a) proved involvement of Activin in the control of digit formation. *Follistatin* expression followed the same temporal and spatial pattern as digit formation, and was coexpressed with *Activin* at the distal growing tip of the digits, suggesting a molecular coordination of Activin signaling, and thus digit formation, by follistatin.

Using FISH techniques, Bondestam *et al.* (1999) localized the human *follistatin* gene to the chromosomal 5q11.2 region. Structurally, follistatin is composed of a signal peptide for secretion, followed by the NH₂-terminal domain and three follistatin domains (FS domains), which consist of ~70 amino acids with 10 conserved cysteine residues (Hemmati-Brivanlou *et al.*, 1994). Shimasaki *et al.* (1988) isolated two forms of *follistatin* cDNA which differed in the 3' region of the open reading frames as a result of alternative splicing of the precursor mRNA. One form encodes a protein of 317 amino acids, while the other form encodes a protein of 344 amino acids, having the same 317 amino acids as the first form but with an additional 27 amino acids at the COOH-terminal side (Table 4). By using linkage and association studies, evidence was provided that the *follistatin* gene was linked with the polycystic ovary syndrome (PCOS; MIM184700), a common endocrine disorder in women, characterized by hyperandrogenism and chronic anovulation leading to female infertility (Urbanek *et al.*, 1999). However, mutation analysis in 34 women diagnosed with PCOS did not result in the identification of disease-causing mutations in the coding sequence of the *follistatin* gene (Calvo *et al.*, 2001) (Table 4).

A number of proteins have been described showing homology to follistatin. Based on their structure, these proteins could be classified in two subfamilies. The first group is the follistatin-related genes (FLRG), like agrin, testican, SPARC, and Flik, which share only one FS domain and do not demonstrate any Activin-binding activity. Flik (follistatin-like) was first isolated from a mouse osteoblastic cell line as a TGF- β 1-inducible protein (called TSC-36) (Shibanuma *et al.*, 1993), and rat and human homologues

were cloned from glioma cell lines (Zwijssen *et al.*, 1994). Studies of the chick *Flik* gene in early embryonic development demonstrated an expression pattern which overlaps with *follistatin* (Patel *et al.*, 1996). From studies in *Xenopus*, follistatin showed a neural-inducing activity (Hemmati-Brivanlou *et al.*, 1994), although *follistatin* null mutant mice did not show any inhibition of neural induction. The loss of follistatin could be compensated noggin and chordin or by a protein structurally related to follistatin. *Flik* was found to be expressed in the ectoderm at the time of neurulation and might therefore be a functional equivalent of *follistatin* and be able to compensate loss of follistatin function in neural induction. Functional studies need to be carried out to verify this hypothesis. The second group of follistatin structural homologues contains at this moment only one protein, which was called follistatin-related protein (FSRP). Hayette *et al.* (1998) first reported the cloning of the mouse and human homologues. Both mouse and human FSRP contain two FS domains, and comparison of the gene structure and overall primary sequence homology between *FSRP* and *follistatin* clearly showed a much greater homology than the other FS domain-containing proteins. Schneyer *et al.* (2001) and Tortoriello *et al.* (2001) were able to demonstrate that FSRP binds to Activin and BMPs in a way similar to follistatin. Despite the structural homology between follistatin and FSRP, both proteins are thought to have distinct biological functions (Schneyer *et al.*, 2001; Tortoriello *et al.*, 2001). Preliminary results from FSRP transgenic mice showed severely impaired fertility relative to control mice (Schneyer *et al.*, 2001).

DAN/Cerberus Protein Family

Recently, a novel family of secreted proteins with antagonistic activities against BMPs was identified, sharing a similar structural motif and similar but nonidentical inductive activities (Hsu *et al.*, 1998; Pearce *et al.*, 1999). The domain of homology in proteins from this family was called the "can-domain," containing the consensus sequence CX₆QX₆CX₆NX₂CXGXCSX₃PX₍₈₋₁₃₎CX₂CXPX₆XLXCX₍₁₅₋₁₉₎CXX (Pearce *et al.*, 1999) (Fig. 3A). Outside this domain, the family members showed little similarity. The domain is related to domains seen in the mucins and the Norrie disease protein (NDP) (Biben *et al.*, 1998) (Fig. 3B) and is structurally related to the cysteine-knot motif of the TGF- β superfamily, with an additional conservation of six amino acid residues (Fig. 3A). Initially, this family contained three proteins: DAN, cerberus, and gremlin, but recently, other proteins were isolated which could be classified as a member of this family: Dte, protein related to DAN and cerberus (PRDC), and also several genes identified as expressed sequence tags in the mouse. Until present, little was known about either the specificity of these antagonists or the biological roles of these proteins. Using *Xenopus* explant assays, Hsu *et al.* (1998) showed that at least DAN, cerberus, and gremlin act as BMP antagonists, presumably in a way similar to noggin and chordin, by

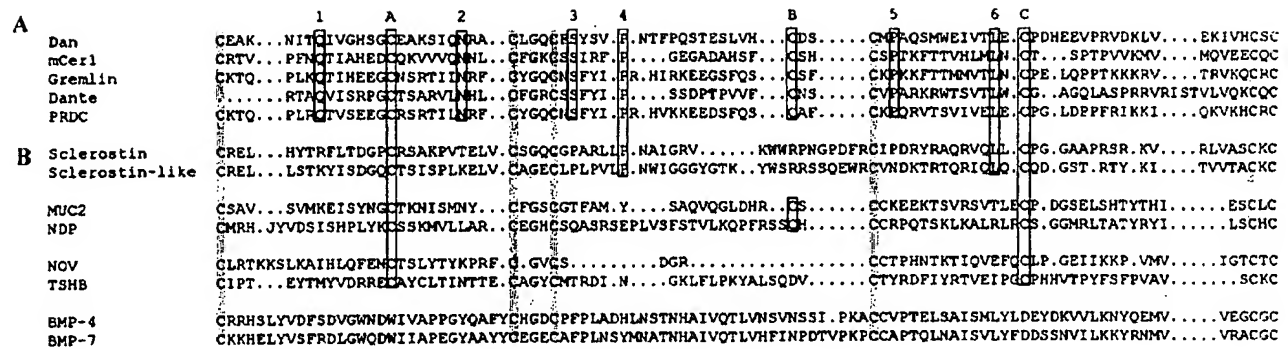


FIG. 3. (A) Alignment of the murine amino acid sequence within the "can-domain" of the different members of the DAN/Cerberus protein family. Conservation of six cysteine and one glycine residues essential for the formation of the core cysteine knot is represented by blue and green bars, respectively. Six additional amino acid residues (Q, N, S, P, P, L) conserved and typical for the can-domain are numbered 1–6. Three additional conserved cysteine residues are numbered A–C. (B) Alignment of murine sclerostin, sclerostin-like, MUC2, NDP, and murine members of the CCN-gene family (NOV), the gonadotropins (TSH-B), and the BMP-family (BMP-4 and BMP-7) to the can-domain.

direct physical binding to BMP molecules and consequently blocking the specific ligand/receptor interactions.

DAN. DAN was originally cloned as a transcript down-regulated in src-transformed rat fibroblasts and was called "differential screening-selected gene aberrant in neuroblastoma" (Ozaki and Sakiyama, 1993). The expression of DAN was found to be significantly reduced in a variety of transformed cells and showed homology to a mouse suppressor gene (Enomoto *et al.*, 1994).

Hsu *et al.* (1998) demonstrated the involvement of DAN in axial patterning activities with the ability to dorsalize ventral mesoderm and neuralize ectoderm in *Xenopus* injection studies, which implies a role for DAN in early gastrulation stages.

During mouse embryogenesis, DAN was expressed in cranial mesenchyme and somites, later in limb and facial mesenchyme. DAN was also expressed in the developing myotome (Stanley *et al.*, 1998; Eimon and Harland, 2001). These expression domains include sites in which BMP inhibition is known to be important for development. DAN expression was seen in virtually all adult tissues, including lung, kidney, brain, intestine, stomach, and spleen. The expression profiles suggest that DAN is a modulator of inductive processes and cell growth during embryonic as well as adult life.

DAN knockout mice were viable, fertile, and did not show any obvious morphological abnormalities in the head mesoderm, somites, facial structures, and limbs during early mouse embryogenesis, nor did these mice show any defects during neural tube development. Skeletal development was also not impaired (Dionne *et al.*, 2001) (Table 2). DAN null mutant mice which were heterozygous for *noggin* displayed, at low penetrance, an apparent transformation on the right-hand side of the last lumbar vertebrae to a sacral fate. This finding suggests that DAN and *noggin* may

be involved in region-specific regulation of BMP and GDF signals in order to properly regulate anterior–posterior identity in the posterior lumbar region (Dionne *et al.*, 2001) (Table 2).

DAN has been shown to bind BMP-2 *in vitro* (Hsu *et al.*, 1998); however, interaction between DAN and any specific TGF- β family member under physiological conditions remains to be demonstrated. Dionne *et al.* (2001) showed that it is unlikely that BMP-2, -4, and -7 are physiological targets for DAN, although overexpressing these BMPs in *Xenopus* embryos did show interaction with DAN. However, they could demonstrate interaction of DAN with GDF-5, another member of the BMP-family and closely related to BMP-2, -4, and -7 (Table 3).

The DAN gene, encoding a secreted protein of 180 amino acids, was mapped to human chromosome 1p36.13-p36.11, in a region known to be involved in genesis/progression of human neuroblastoma (Enomoto *et al.*, 1994) and was considered as a possible candidate for a tumor suppressor gene of human neuroblastoma. However, loss of heterozygosity (LOH) studies excluded DAN as a candidate gene (White *et al.*, 1995) (Table 4).

Cerberus. Cerberus is a secreted growth factor expressed in the Spemann organizer and anterior endomesoderm of gastrula-stage embryos. Whereas *noggin*, chordin, follistatin, and DAN are able to induce neural tissue development and axis formation when mRNA is injected into *Xenopus* embryos, Cerberus suppresses the formation of posterior mesoderm and specifically induces formation of ectopic head structures, such as cement gland, olfactory placodes, eyes, and forebrain (Bouwmeester *et al.*, 1996). Biben *et al.* (1998) suggested that *Xenopus* Cerberus (xCer), which showed BMP-4, Nodal, and Wnt antagonistic activities, might participate directly or indirectly in freeing the prospective head region from the influence of these ventral-

izing growth factors, a theory which has been confirmed by Piccolo *et al.* (1999). Additionally, xCer was able to antagonize the activity of the mesoderm-inducing Activin ligands (Hsu *et al.*, 1998) (Table 3).

Shawlot *et al.* (1998) described a mouse homologue of xCer and called this gene mouse cerberus-related gene *mCer1*, also known as *Cerr1*, *cer-1*, *Cer1*, with the *mCer1* protein sharing 57% similarity to xCer in the C-terminal cysteine-knot region. Although the expression patterns of xCer and *mCer1* overlap in early embryogenesis (Belo *et al.*, 1997; Biben *et al.*, 1998), it is still unclear whether *mCer1* is the mouse homologue of xCer or only a *Cerberus* gene family member. During early mouse embryogenesis, *mCer1* is expressed in the anterior visceral and definitive mesoderm and in the forming somitic mesoderm (Shawlot *et al.*, 1998), suggesting a role in development of the axial skeleton, musculature, and peripheral nervous system. The neural-inducing and mesoderm-inhibiting activities of *mCer* in mice result from specific binding to BMP and Nodal proteins, respectively. However, in contrast with xCer, no direct inhibition of Wnt signals was detected (Belo *et al.*, 2000). Thus far, evidence for binding of *mCer* to Activin has also not been provided. No expression of *mCer1* was detected in adult tissues, such as brain, skeletal muscle, heart, lung, stomach, spleen, liver, kidney, uterus, testis, and ovary (Biben *et al.*, 1998). Simpson *et al.* (1999) studied an inbred mouse strain carrying a chromosomal deletion encompassing *mCer1*, which was called *Tyrp1^{h-461THC}*. *mCer1*-deleted mouse embryos have grossly normal morphology, and in particular they had developed normal head structures. Anterior patterning and somite formation was not disrupted. These results demonstrated that *mCer1* is not required for normal morphological development of the mouse embryo and they suggest that other proteins with overlapping functions may be capable of compensating *mCer1* function (Table 2). Furthermore, Belo *et al.* (2000) generated a *mCer1* null allele by targeted inactivation in ES cells. The homozygous knockout mice showed no anterior patterning defects, are born alive, and are fertile (Table 2). Shawlot *et al.* (2000) also generated *mCer1*-deficient mice by targeted inactivation in ES cells. Comparison with wild type embryos revealed no obvious defects in head formation in *mCer1^{-/-}* embryos, and also heart development and somite formation appeared normal. At later stages of embryonic development, the null mice appeared phenotypically identical to their normal littermates. Skeletal development was also normal in newborn *mCer1^{-/-}* mice (Table 2). Stanley *et al.* (2000) generated *mCer1* null mice by targeted insertion of a *lacZ* reporter gene in the *mCer* locus, which allowed them to study *mCer1* expression patterns during embryonic and adult development. Besides previously reported expression profiles of *mCer1* at sites of embryonic development (Shawlot *et al.*, 1998; Biben *et al.*, 1998), they showed the presence of *mCer1* in the developing lung, pancreas, hair follicles, and nervous system. In the developing skeleton, *mCer1* was seen in areas of new cartilage formation, in the developing ear, presumptive limb joints, vertebra, and ribs. Postnatally, expression was associated with

numerous structures around the joint. Development at these sites of *mCer1* expression appeared normal in *mCer1^{lacZ/lacZ}* mutants (Table 2). Results from expression studies within these four knockout mice models suggested that *mCer* is not essential in mouse development and that *mCer1* function might be compensated by another molecule. Borges *et al.* (2001) studied a *mCer1^{-/-}/noggin^{-/-}* double knockout mouse model to check possible compensation, but failed to show any additional detectable defects besides the ones presented by the *noggin^{-/-}* single mutants (Table 2).

Expression of *Cerberus* in the chick (*cCer*) showed an overlapping expression pattern with *Nodal*, a gene involved in left-right asymmetry of the embryonic head (Zhu *et al.*, 1999b). *cCer* was found to be restricted to the left lateral plate mesoderm and head mesenchyme. The left-sided expression of *cCer* was found to be regulated by *Sonic Hedgehog* (*Shh*), both in the head and trunk; expression of the left side in the head, but not in the trunk, was also controlled by *Nodal* (Zhu *et al.*, 1999b; King and Brown, 1999). Yokouchi *et al.* (1999) isolated a novel *cerberus*-related gene, called *Caronte*, from the chick, which showed antagonistic activities against BMP-2, -4, and -7, Activin, and Nodal, and with asymmetrical expression in the left paraxial mesoderm. *Caronte* is induced by *Shh* and repressed by FGF8, and plays a role in Nodal signaling. Functional and expression analyses of *Caronte* showed identical patterns with *cCer* and sequence comparison of the two proteins showed complete homology, indicating that both proteins are the same.

The human *Cerberus* gene (*CER1*) was isolated by library screening and localized by radiation hybrid mapping to chromosome 9p22 (Lah *et al.*, 1999). The *CER1* protein contains 267 amino acids with a signal peptide for secretion. Thus far, no disease phenotype is associated with mutations in the *CER1* gene (Table 4).

Gremlin. Using a differential expression assay, Topol *et al.* (1997) isolated *gremlin* or *drm* (down-regulated in transformed cells), showing potential roles in cell growth and tissue-specific differentiation. Expression of *gremlin* could not be detected in a vast majority of malignant cell lines, supporting the hypothesis that *gremlin* might play an important role in carcinogenesis, functioning as an inhibitor of tumor progression.

Hsu *et al.* (1998) identified *Xenopus gremlin* after a screen for molecules capable of inducing a secondary axis in the *Xenopus* embryo. *Gremlin* is not expressed during early gastrulation of *Xenopus* embryos, despite axial patterning activities, suggesting that its contribution to pattern formation will be in later steps. Instead, expression begins at tailbud stages, where it is correlated with neural crest lineages. Later on in development, *gremlin* expression is extended rostrally and caudally to include neural crest cells at all axial levels, suggesting a role in neural crest induction and patterning.

Expression of *gremlin* during mouse embryogenesis starts at the somite stage and the anterior ventral forebrain in the neural tube. Later on, *gremlin* was observed in the dorsal

and ventral mesenchyme of the limb buds, first in the dorsal proximal domain and later in the proximal interdigital regions (Pearce *et al.*, 1999).

By comparing *gremlin* expression patterns in chick embryos with those seen in *Xenopus* (Hsu *et al.*, 1998) and mouse (Pearce *et al.*, 1999), significant differences were observed. In the chick, *gremlin* transcripts were detected in the limb buds, the central part of branchial arches I, II, and III, with strong expression in the mandibular and maxillary processes of branchial arch I. Later on in chick development, some expression was seen on both sides of the neural tube in the trunk region, corresponding to the caudal part of each myotome. Finally, *gremlin* expression was found in the lateral part of the dermatome (Bardot *et al.*, 2001).

Gremlin plays a role as mediator during vertebrate limb outgrowth, in both mouse (Zuniga *et al.*, 1999) and avian (Merino *et al.*, 1999b), where it is coexpressed with BMP-2, -4, and -7, secreted factors known to play important roles in limb development. Outgrowth and patterning of the vertebrate limb are controlled by reciprocal interactions between the posterior mesenchyme (polarizing region) and a specialized ectodermal structure, the apical ectodermal ridge (AER). Sonic hedgehog (Shh) and fibroblast growth factors (FGFs) are key signaling molecules produced in the polarizing region and AER, respectively. Shh signaling by the polarizing region modulates FGF signaling by the AER, which in turn mediates the polarizing region (Shh/FGF4 feedback loop). By antagonizing BMP repression of FGF activity, gremlin regulates this Shh/FGF4 feedback loop (Zuniga *et al.*, 1999). The ability of gremlin to bind and block BMP-2, -4, and -7 activity has been demonstrated both *in vivo* and *in vitro* (Hsu *et al.*, 1998; Merino *et al.*, 1999b) (Table 3).

Gremlin is not only involved during early embryogenesis, expression is also highly regulated in various adult rat tissues, with high levels of expression in brain, spleen, kidney, and testis, and it is particularly expressed in terminally differentiated cells, suggesting specific roles and functional mechanisms in adult tissues (Topol *et al.*, 1997, 2000a).

Radiation hybrid mapping was used to localize the human *gremlin* gene to chromosome 15q13-q15, in a region where association with human malignancies has been reported (Topol *et al.*, 2000b). The human *gremlin* cDNA encodes a predicted protein of 184 amino acids containing a signal peptide for secretion (Table 4). Bacchelli *et al.* (2001) suggested that *gremlin* might be mutated in patients with the autosomal recessive Cenani-Lenz syndrome (CLS; MIM212780). The limb abnormalities in CLS closely resemble the ones found in a recessive mouse mutant, *limb deformity (ld)*, mutated in the *formin* gene (Woychik *et al.*, 1990; Mass *et al.*, 1990). Gremlin is a downstream target of formin and its expression is known to be lost in *ld* mice (Zuniga *et al.*, 1999). However, haplotype analysis in one CLS patient with consanguineous parents using genetic markers derived from the chromosome 15q13-q15 region

excluded *gremlin* as a candidate gene for CLS (Bacchelli *et al.*, 2001).

Dante. Based on structural homology, Pearce *et al.* (1999) identified Dante (Dte), a novel DAN/Cerberus family member in the mouse. However, screening assays for full-length *Dte* cDNA has not yet been successful. The amino acid sequence of Dte showed absence of the conserved amino-terminal cysteine residue (Fig. 3A), but according to Pearce *et al.* (1999), this could probably be due to cloning difficulties. Keeping these concerns in mind, *Dte* could be considered as a new gene because of sequence specificity and expression profiles. At this moment, very little is known about functional activities of this protein, but expression patterns of *Dte* in mice suggest a potential role during early stages of mouse embryonic development. *Dte* expression was first observed in the definitive node at the early bud stage in a pattern similar to that of *Nodal*, a protein required for primitive streak formation and involved in left-right asymmetry. By early somite stage, strongest *Dte* expression was seen on the right-hand side of the node, in contrast with later expression of *Nodal*, which is expressed on the left-hand side of the node. Later on in mouse embryogenesis, *Dte* expression was no longer observed (Pearce *et al.*, 1999).

Further experiments need to be carried out to study possible binding to members of the BMP family and to find out whether Dte is able to inhibit BMP signaling. Pearce *et al.* (1999) did already hypothesize a possible role for Dte in antagonizing *Nodal*, whereas initially, *Dte* and *Nodal* are expressed symmetrically, but by early somite stage, expression of both genes becomes biased in a way that *Dte* expression is highest at the right, while *Nodal* is stronger at the left.

PRDC. Using a gene trap approach in ES cells to identify genes involved in mouse development, Minabe-Saegusa *et al.* (1998) identified a novel mouse gene, *PRDC* (protein related to DAN and Cerberus), which showed limited similarities to DAN and Cerberus. Within the can-domain, PRDC showed a high degree of sequence similarity with gremlin, another member of the DAN/Cerberus family (Fig. 2), suggesting possible overlapping or complementary functions. Although PRDC belongs to the DAN/Cerberus family based on its structure, thus far, no evidence has been provided that PRDC can bind and antagonize members of the BMP family.

During experimental procedures of the gene trapping, homozygous mice carrying part of the *PRDC* gene downstream of the *lacZ* gene were generated, which allowed studying expression patterns of *PRDC* during mouse development. The reporter gene expression was detected in commissural neurons in the developing spinal cord, suggesting a role for PRDC during mouse neural development. Expression analysis of endogenous *PRDC* in these mice is consistent with patterns seen from the *PRDC/lacZ* reporter gene (Minabe-Saegusa *et al.*, 1998).

Homology searches using the BLAST algorithm (Altschul *et al.*, 1990) with the mRNA sequence of the human *PRDC*

gene, encoding a secreted protein of 168 amino acid residues, assigned the gene to human chromosome 1q42-q43 on genomic clone RP11-467I20 (GenBank Accession No. AL358176). To date, no disease has been identified with mutations in the *PRDC* gene (Table 4).

Sclerostin

Using a positional cloning strategy to identify the disease-causing gene in sclerosteosis, a sclerosing bone dysplasia characterized by a massive bone overgrowth, a new gene, *SOST*, was isolated which encodes a protein, sclerostin, of 213 amino acid residues, with a signal peptide for secretion (Balemans *et al.*, 2001; Brunkow *et al.*, 2001). Amino acid sequence analysis demonstrated the conservation of six cysteine and one glycine residues, which are essential to form the core cysteine knot, and of two additional cysteines also seen in the DAN/Cerberus protein family, the mucins, NDP, the CCN-gene family, and the gonadotropins (Figs. 3A and 3B). However, based on amino acid homology, this protein does not belong to the DAN/Cerberus protein family, because of lack of conservation of four of the six amino acid residues typical for the can-domain. Additionally, the conservation of one cysteine residue, which is found in the DAN/Cerberus protein family and also in the mucins and NDP, is lacking in sclerostin (Figs. 3A and 3B). Therefore, we can classify this protein as a novel member of the cysteine-knot superfamily of growth factors, but not within the DAN/Cerberus protein family.

During mouse embryogenesis, *SOST* expression was detected in the neural tube, limb buds, blood vessels, and ossifying cartilages in mandible, cervical vertebra, occipital bone, ribs, and palate, suggesting a possible role of sclerostin during mouse development (unpublished data; patent PCT/US99/27990). In adult human tissues, the expression pattern was restricted to kidney, bone marrow, and osteoblasts differentiated for 21 days, implicating a very select role for sclerostin in adult life (Balemans *et al.*, 2001). *In situ* hybridization experiments on human kidney sections using a *SOST* antisense RNA probe showed a restricted expression in the glomeruli (unpublished data).

In vitro studies demonstrated binding of sclerostin to the closely related BMP-5 and BMP-6 (Table 3), but not to BMP-4 (unpublished results; patent PCT/US99/27990); however, evidence needs to be provided that sclerostin acts as an antagonist for BMPs under physiological conditions. The structure of sclerostin, with the presence of the cysteine-knot, suggests direct binding of sclerostin to BMPs in a manner similar to members of the DAN/Cerberus protein family, and in this way interfering directly with BMP signaling by inhibiting the binding to the BMP receptors.

The human *SOST* gene is located on chromosome 17q12-q21, in a region where both sclerosteosis (*SOST*; MIM-269500) (Balemans *et al.*, 1999) and van Buchem disease (VBCH; MIM239100) (Van Hul *et al.*, 1998) are located. Both conditions are craniotubular hyperostoses and display with

similar skeletal abnormalities of a generalized hyperostosis (Van Buchem *et al.*, 1962; Truswell, 1958). Sclerosteosis showed additional clinical features, such as a tall stature and hand malformations (Beighton *et al.*, 1984); however, both conditions are thought to be allelic (Beighton *et al.*, 1984). Loss-of-function mutations in *SOST* were found in patients with sclerosteosis (Balemans *et al.*, 2001; Brunkow *et al.*, 2001), and the presence of a deletion 35 kb downstream of *SOST* in patients with van Buchem disease suggests that this condition is caused by inappropriate regulation of *SOST* expression (Balemans *et al.*, 2002) (Table 4).

Homology searches with the mRNA and protein sequences of sclerostin showed weak homology with a new gene, referred to as *SOST-like*, located on human chromosome 7p21. This gene encodes a secreted protein, *sclerostin-like*, containing 206 amino acid residues with a cysteine knot motif, and also lacking the characteristics of the can-domain (Fig. 3B). An amino acid homology between sclerostin and *sclerostin-like* of 36% is observed. The identification of these two homologous proteins may suggest the existence of a novel protein family of secreted growth factors. Further experiments need to be carried out to elucidate possible roles of these proteins during development and in adult tissues.

DISCUSSION AND PERSPECTIVES

The past few years have been exciting for the BMP field, due to the identification of a class of proteins which modulate BMP signaling. It is now becoming increasingly clear that many aspects of development in vertebrates and invertebrates are regulated by opposing activities of secreted ligands and their extracellular antagonists, which together define the tightly regulated spatiotemporal domains of target activation. In this paper, we reviewed the current state of the extracellular modulators of BMP signaling in vertebrates. To date, nine extracellular proteins with BMP binding capacities—noggin, chordin, chordin-like, follistatin, FSRP, DAN, Cerberus, gremlin, and sclerostin—have been identified, three of which belong to a distinct protein family: the DAN/Cerberus family. Additionally, three proteins show significant homology to at least one of them—Dte, PRDC, and *sclerostin-like*. For a subset of these BMP binding molecules, it is known that they bind BMP family members with different degrees of specificity (Hsu *et al.*, 1998), and the activities of a large number of these proteins suggest that they antagonize BMP signaling using a similar mechanism. This hypothesis is supported by the finding that noggin, chordin, follistatin, DAN, Cerberus, and gremlin all bind BMP-2, -4, and -7 (Table 3). Furthermore, noggin efficiently competes with DAN/Cerberus family members for binding to BMP-2, suggesting that the interaction between BMP-2 and these antagonists occurs through a similar BMP domain (Hsu *et al.*, 1998).

For a number of BMP antagonists, it is known that their

regulation of BMP signaling is not a one-way process. Feedback mechanisms exist whereby members of the BMP family modulate their own activity. Distal outgrowth of the chick limb is, in part, controlled by a regulatory loop between BMPs and gremlin (Merino *et al.*, 1999b). Induction of noggin has been observed in cultured rat osteoblasts and mouse forelimb organ cultures treated with BMP-2 and BMP-7 (Gazzerro *et al.*, 1998; Nifuji and Noda, 1999). *Noggin* expression is also upregulated after incubation of developing chick somites with BMP-4 (Arnthor *et al.*, 1999), and ectopic expression of *folistatin* was seen in the developing chick limb bud after exogenous administration of GDF-11 (Gamer *et al.*, 2001).

Different findings support the hypothesis that different BMP antagonists might exhibit overlapping and/or complementary functions during development. For example, in early embryogenesis, *noggin* and *chordin* single knockout mice did not show any developmental defects, while *noggin/chordin* double knockouts displayed numerous defects during gastrulation, suggesting that both proteins might be functional equivalents during these early stages of development (Bachiller *et al.*, 2000).

Studies of expression patterns and mouse models of BMP antagonists provided huge amounts of information on the involvement of these molecules in major processes of development. A key role of the BMP antagonist activities resides within the organizer during the first stages of embryonic development, where they are implemented in the induction and patterning of ventral mesoderm. Several studies in *Xenopus* and *Drosophila* have demonstrated that the mechanism of dorsal-ventral patterning in both vertebrate and invertebrate species shares a common plan, whereby a conserved system of antagonistic extracellular signals, i.e., *sog*-*chordin*/*noggin* opposed the action of their ligands, i.e., *dpp*/*screw*-BMP-2/-4 (Padgett *et al.*, 1993; Schmidt *et al.*, 1995; Holley *et al.*, 1995, 1996). In vertebrates, ventral cell fates are specified by the action of BMPs and are antagonized by dorsally expressed antagonists. In invertebrates, the same relationship exists between *dpp*/*screw* and *sog*, but the axis is inverted. A number of vertebrate BMP antagonists, such as *noggin*, *chordin*, *folistatin*, and *DAN*, are expressed nearby in the organizer and, due to their dorsalizing activities, can act as a defense mechanism against being ventralized (reviewed in Thomsen, 1997). Another important, but still not fully understood, BMP antagonist function is the induction of neural tissue from the ectoderm and is today a major focus of interest for embryologists. BMP signals are known to pattern ectoderm during gastrulation, by inducing epidermis from ectoderm which would otherwise transform into a neural state (Sasai *et al.*, 1995). The identification of different BMP ligands and their counteracting neural fate-inducing BMP antagonists has led to the establishment of the "default model" of neurulation in the *Xenopus* embryo (Hemmati-Brivanlou and Melton, 1997). To date, five BMP antagonist molecules are known to be involved in neural induction in vertebrates: *noggin*, *chordin*, *folistatin*, *DAN*,

and *Cerberus*. However, it is now also known that BMP inhibition by these antagonists is not sufficient for neural induction, but rather, acts downstream of, or in conjunction with, other signals from the organizer. Several groups are currently investigating the involvement of the FGF (fibroblast growth factor) and Wnt pathways in specifying neural fates. It has been shown that both pathways participate in neural induction, either directly or indirectly through BMP signaling (Lamb and Harland, 1995; Launay *et al.*, 1996; Gomez-Skarmeta *et al.*, 2001; Wilson *et al.*, 2001). Although the knowledge about the evolutionary conservation of neural induction among vertebrates and invertebrates is still confusing, several recent experimental findings have tackled the hypothesis of a common plan for neural induction, because of differences in the time and in the mechanism of specification of neural cells between different species, while other groups propose a possible unifying mechanism (reviewed in Wilson and Edlund, 2001; Muñoz-Sanjuán and Brivanlou, 2002). Studies in *Xenopus* and zebrafish clearly indicated a role for several BMP antagonists in neural induction; however, single knockout mouse models of these neural-inducing antagonists did not reveal any defects in neural induction during early gastrulation (Table 2). These findings could be explained by the existence of proteins which are able to rescue the neural induction process. The generation of multiple (double, triple, quadruple, or greater combinations) knockout models, in which different BMP antagonists or a combination of BMP antagonists and members of the Wnt and FGF pathways are knocked out, may provide better insights in the processes of neural induction. Zuniga *et al.* (1999) and Merino *et al.* (1999a,b) described how antagonistic interactions of *noggin*, *folistatin*, and *gremlin* coordinate the vertebrate limb proximal-distal specification and the control of limb bud outgrowth and proliferation by altering BMP signaling. Besides these three major functions, BMPs and their antagonists are known to be involved in numerous other processes, indicating the importance of this control system during different phases of development and in the patterning and maintenance of adult tissues.

When considering the increasing number of BMP antagonists discovered, and the identification of homologues of the different BMP antagonists, such as *chordin-like*, *FSRP*, and *sclerostin-like*, it may be clear that these molecules act in a complex cascade of tightly regulated actions in the processes of vertebrate and invertebrate patterning. However, a lot of research still needs to be carried out to completely solve and understand the entire process of embryonic development and the involvement of these antagonists in adult tissues.

ACKNOWLEDGMENTS

This work was supported by Interuniversity Attraction Pole grant (to W.V.H.) and a grant (G.0404.00) from the "Fonds Voor Wetenschappelijk Onderzoek (FWO)" (to W.V.H.).

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Received for publication March 6, 2002

Revised June 20, 2002

Accepted June 22, 2002

Published online August 26, 2002